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Content of N-6 methyl adenylic acid in heterogeneous nuclear and messenger RNA of HeLa cells

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ABSTRACT

With the aid of a suitable thin layer chromatographic procedure, the N-6 methyl adenylic acid (m^6A), content of a variety of ^{32}P labeled RNA species from HeLa cells has been measured.

Poly(A)-containing (poly(A)+) cytoplasmic RNA has on the average one m^6Ap per 800 to 900 nucleotides. This value is independent of the length of the molecules. The proportion of m^6Ap in poly(A)+ cytoplasmic RNA does not change between 4 and 18 hours of labeling with ^{32}P, suggesting that the majority of the messenger RNA molecules may have a similar level of internal methylation regardless of their half-life. The non-polyadenylated, non-ribosomal cytoplasmic RNA fraction sedimenting from 10S to 28S is less methylated with approximately one m^6A per 2,700 nucleotides.

Heterogeneous nuclear RNA molecules (DMSO treated) which sediment from 28S to 45S have approximately one m^6Ap per 3,000 nucleotides. The hnRNA molecules sedimenting from 10S to 28S have one m^6Ap per 1,800 nucleotides. Poly(A)+ nuclear RNA is enriched in m^6A, containing 1 residue of m^6A per 700 to 800 nucleotides, a value close to that obtained for the polyadenylated cytoplasmic RNA.

INTRODUCTION

N^6 methyl adenylic acid (m^6Ap) is the major internal methylation found in most of the messenger RNA (mRNA) and heterogeneous nuclear RNA (hnRNA) of animal cells and of the DNA viruses that replicate in the nucleus (1). m^6Ap is absent from mRNA of lower eukaryotic cells such as yeast (2) and slime molds (3), as well as from animal viruses that replicate in the cytoplasm

(1). Previous estimations of m^6Ap content were made relative to methylation in the m^7G-containing 5' terminal oligonucleotide termed "caps", by comparing the incorporation of methyl groups from ^3H methionine (4) into each. The accuracy of earlier estimation depends upon the assumption that there is no variation in the specific activity of the pools for different methylations and that there is no significant delay between the time of the methylation of different structures. Therefore in this work we measured m^6Ap directly by thin layer chromatography of RNAse digests of poly(A) terminated and non-poly(A) terminated RNA molecules of HeLa cells.

RESULTS

m^6Ap Analysis: Cells were labeled with (^{32}P) phosphate and various RNA fractions extracted and digested to mononucleotides with pancreatic and T2 RNAses as described in Methods. Nishimura (5) developed a two dimensional thin-layer chromatographic procedure for the separation of modified mononucleotides that separates m^6Ap from most of the other nucleotides. This procedure involves an acid solvent (isopropanol:HCl:H_2O, pH 2.5) which causes depurination and might interfere with the evaluation. We have therefore modified Nishimura's second solvent to isopropanol-NH_4OH-H_2O 10:1:30 (v/v) (6). With this modification m^6Ap is well separated from the other mononucleotides (Plate 1). The cap structures chromatographed more slowly in the second dimension and were masked by the four mononucleotides. The four small spots in the center of the plate correspond to the 2'-3' cyclic mononucleotides generated during the pancreatic and T2 RNAses digestion. Any conversion of possible m^1Ap to m^6Ap, due to the high pH of the isopropanol-NH_3 solvent would not interfere with this evaluation since m^1Ap is well separated from m^6Ap during the first dimension. Thus this procedure can be used for detection and quantitation of a very small proportion of m^6Ap and is suitable for the analysis of m^6dAp in DNA where otherwise the depuration is complete (unpublished observation).

CONTENT OF m^6Ap IN hnRNA AND mRNA

hnRNA was labeled with ^{32}P for 4 hours. The hnRNA was either chromatographed on poly(U) sepharose and poly(A)+ and poly(A)- fractions separated, or directly denatured in 95% DMSO and then sedimented on sucrose gradients (see Methods). The total

hnRNA poly(A)+ fraction had a larger average size than mRNA (in agreement with previous results) (7), both by sedimentation analysis (not shown) and by the fact that they contained 3% of their radioactivity in poly(A) tracts of adenylic acid residues indicating an average size of 5000-6000 nucleotides (data not shown).

The cytoplasmic RNA molecules labeled for 4 hours or 18 hours with ^{32}P were obtained from the postmitochondrial cytoplasmic fraction separated into size classes by sucrose zonal sedimentation and a poly(A)+ and poly(A)- (8) fraction obtained from each size class by poly(U) sepharose chromatography.

The estimation of m^6Ap in these fractions is shown in Table 1, the main features of the methylation are the following:

1. Poly(A)+ cytoplasmic RNA contains 1 m^6Ap per 800-900 nucleotides regardless of length and hnRNA larger than 28S contains about one residue per 3,000 nucleotides.
2. The average content of m^6Ap in poly(A) + nuclear RNA is similar to that of cytoplasmic RNA poly(A)+ with one residue per 700 to 800 nucleotides.
3. The polyadenylated hnRNA and cytoplasmic RNA have on the average about three or four times more m^6Ap compared to the non-polyadenylated molecules of the same size.
4. The content of m^6Ap in the different sized molecules of cytoplasmic RNA poly(A)+ is the same after 4 or 18 hours of labeling the cells with ^{32}P phosphate.

DISCUSSION

These data indicate that nuclear RNA larger than 28S has one m^6Ap per approximately 3,000 nucleotides and this ratio does not change with the size of the molecules. The 18S to 28S RNA contains 1 to 2 m^6Ap per molecule while those molecules sedimenting faster than 45S contain an average of 5 or more m^6Ap residues. These values are similar to the indirect determinations for hnRNA molecules of HeLa cells (4) and L cells (9). Considering that m^6Ap is not located in the poly(A) sequences themselves (4,10) and that poly A+ hnRNA and mRNA have more m^6Ap than polyA- fractions it appears that the two events (poly(A) addition and methylation of m^6Ap) occur frequently in the same group of nuclear molecules.

The constant average m^6Ap content of poly(A)+ cytoplasmic

TABLE I

Exp.	^{32}P RNA	% ^{32}P in Poly(A)	Size(s)	cpm in mono-nucleotides	m^6Ap	Average number of nucleotides per one 6mAp
I	HnRNA	0.06%	>45S	1,717,170	510	3,367
	HnRNA	0.07%	45S	1,255,180	388	3,235
	HnRNA	0.4%	32S	6,603.100	1,997	3,300
	HnRNA	0.1%	18S-28S	2,017,050	1,130	1,785
II	HnRNA Poly(A)+	3.4%	>18S	118.990	169	705
III	cRNA Poly(A)+	7.6%	10S-28S	903,270	1,115	810
	cRNA Poly(A)-	0.2%	10S-28S	514,970	189	2,725
IV	cRNA Poly(A)+	6.6%	28S	210,480	240	877
	cRNA Poly(A)+	7.8	18S-28S	788,000	816	965
	cRNA Poly(A)+	8.1	10S-18S	253,700	270	939
V	4h labeled cRNA Poly(A)+		10S-20S	1,222,545	1,480	826
	4h labeled cRNA Poly (A)+		20S-28S	1,333,560	1,806	740
VI	18h labeled cRNA Poly(A)+	3.4%	28S	207,396	185	1,120
	"	5.0%	18S-28S	602,846	585	1,030
	"	5.9%	10S-18S	198,825	175	1,135

Content of m^6Ap in different hnRNA and cytoplasmic RNA fractions. The background radioactivity of about 25 CPM for each determination has been subtracted. If not indicated, the ^{32}P labeling time was 4 hours. See methods for preparation of the sample and the chromatography procedure.

RNA (1/800-900 nucleotides) regardless of size signifies from 1 to 5 residues per molecule in the 10S to 28S size range, and might indicate an increased frequency of a particular base sequence(s) the longer the mRNA. The fact that the level of m^6Ap does not change appreciably from 4 to 18 hours for the polyadenylated cytoplasmic RNA indicates that the majority of the mRNA has a similar level of internal methylation independently of its half life.

The poly A(+) HnRNA and mRNA molecules probably contain the same frequency per unit length of sequences susceptible to the methylation of adenine in the 6 position (11).

MATERIAL AND METHODS

The procedures for growing, labeling and extracting hnRNA and mRNA from HeLa cells treated with actinomycin to suppress rRNA synthesis have already been described (12). For the 4 hours label Actinomycin D was added at a concentration of 0.05µg per ml 30 minutes before the ^{32}P. In the 18 hours ^{32}P label experi-

ments no actinomycin D was added and after 4 hours of labeling in phosphate-free medium cold inorganic phosphate was added to a concentration of $5x10^{-5}M$.

To fractionate the RNAs by size both cytoplasmic and nuclear RNA fractions were made 95% in DMSO and then sedimented in sucrose gradients as described (7). The poly(A) sequence content and length were determined after digestion with T1 RNAse by poly(U) sepharose selection and acrylamide gel (15%) electrophoresis as described (4). Complete digestion of RNA was carried out by boiling in 10 mM Tris HCl pH 7.6, 10 mm EDTA for 3 minutes, quick cooling and digestion with pancreatic and T2 RNAses as described (4). Either the total digestion mixtures in volumes smaller than 4 µl or the mononucleotide fraction taken from a DEAE cellulose column (13) were fractionated by two dimensional thin-layer chromatography for m^6Ap determination.

The chromatography was carried out on 500 µ thick microcrystalline cellulose 20x20 cm plates; the capacity was about 50 µg of digested RNA. The first dimension used was Isobutyric acid - 0.5 M NH_3 in a ratio of 5:3 (v/v) (Nishimura) (5). The front was allowed to reach the top of the plate. After drying up the plate at room temperature with an air blower, the second dimension was developed at 90° with Isopropanol: concentrated ammonia:water, 70:1:30 (v/v) (6) until the front reached the top of the plate. The approximate time for each dimension was from 4 to 6 hours. The plates were dryed up wrapped in plastic film and autoradiographed for the time necessary to locate the m^6Ap (Rf 1st = 0.70 Rf 2nd = 0.34) as shown in Fig. 1. Alternatively an unlabeled marker of 3' m^6Ap could be added to the sample and the spot located using a UV light. The m^6Ap and four nucleotide spots were scraped carefully from the plate cutting out the film and the powder sucked into small disposable plastic syringes stopped with glass wool and connected to vacuum. The contents of the syringes were eluted into vials and three 1 ml portions of water and the radioactivity measured directly by Cherenkov radiation or after addition with a Triton X-100 toluene fluor mixture with 50 to 60% efficiency.

Materials: T_2 RNAse was obtained from Sankyo, Japan; pancreatic RNAse from Calbiochem. ^{32}P carrier free phosphate from

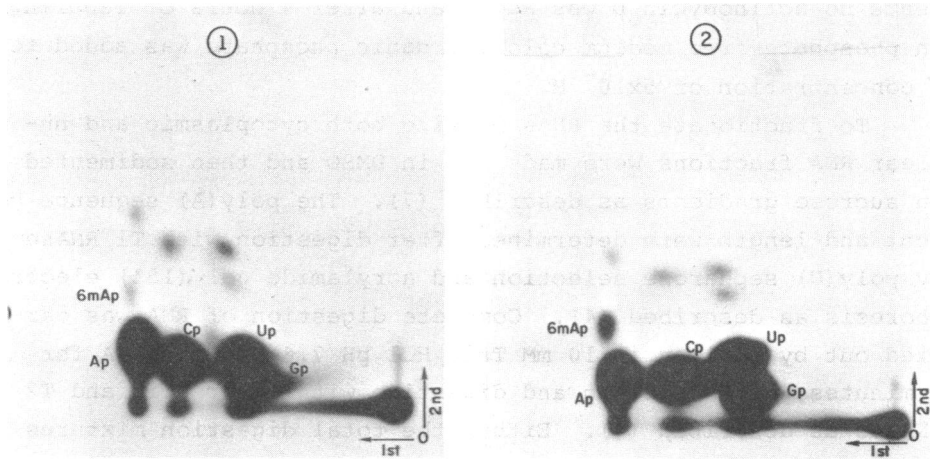

Figure 1.
Autoradiogram from a typical thin layer chromatogram of a T2 RNAse digestion of hnRNA (1) and mRNA (2) showing the separation of m^6Ap from the remainder of the mononucleotides. See methods for preparation of the sample and the chromatography procedure.

New England Nuclear. 500 μ thick microcrystalline cellulose 20x20 cm plates from Analtec. Isobutyric acid from Kodak and Isopropanol analytical reagent from Fisher.

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